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(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Clear, Chemically Modified Collagen-Synthetic Polymer  
Conjugates for Ophthalmic Applications

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Notice: This application is as filed and may therefore contain an  
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1 CLEAR, CHEMICALLY MODIFIED COLLAGEN-SYNTHETIC POLYMER  
2 CONJUGATES FOR OPHTHALMIC APPLICATIONS

3 Abstract of the Disclosure

4 Various forms of chemically modified collagen are covalently crosslinked with activated  
5 synthetic hydrophilic polymers to form optically clear biocompatible conjugates useful in a variety  
6 of medical applications, particularly in ophthalmic devices. The chemically modified collagen is in  
7 substantially nonfibrillar form at pH 7 and is preferably succinylated or methylated collagen. The  
8 synthetic hydrophilic polymer is preferably an activated polymeric glycol, most preferably, a di- or  
9 multifunctionally activated polyethylene glycol. Materials and devices formed with the chemically  
10 modified collagen-synthetic polymer conjugates have good optical clarity, mechanical strength, and  
11 moldability.

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CLEAR, CHEMICALLY MODIFIED COLLAGEN-SYNTHETIC POLYMER  
CONJUGATES FOR OPHTHALMIC APPLICATIONS

Cross-References

This application is a continuation-in-part of copending U.S. application Serial No. 07/922,541, filed July 30, 1992, which is a continuation-in-part of U.S. Patent No. 5,162,430, issued November 10, 1992, which is a continuation-in-part of U.S. application Serial No. 07/274,071, filed November 21, 1988, subsequently abandoned, which applications and issued patents are incorporated herein by reference in full and to which we claim priority under 35 USC §120.

Field of the Invention

This invention relates to biocompatible conjugates comprising chemically modified collagens, which are in substantially nonfibrillar form at pH 7, covalently bound to activated synthetic hydrophilic polymers, which conjugates are formulated to be optically clear and have superior mechanical strength compared to non-crosslinked formulations, which make them suitable for use in ophthalmic devices.

Background of the Invention

Daniels et al, U.S. Pat. No. 3,949,073, disclosed the preparation of soluble collagen by dissolving tissue in aqueous acid, followed by enzymatic digestion. The resulting atelopeptide collagen is soluble, and substantially less immunogenic than unmodified collagen. It may be injected into suitable locations of a subject with a fibril-formation promoter (described as a polymerization promoter in the patent) to form fibrous collagen implants *in situ*, for augmenting hard or soft tissue. This material is now commercially available from Collagen Corporation (Palo Alto, CA) under the trademark Zyderm® Collagen Implant.

Miyata et al, U.S. Pat. No. 4,164,559, disclosed an ophthalmic drug delivery system comprising a chemically modified collagen thin membrane carrier. While these materials are clear, they do not have the mechanical strength required for ophthalmic devices such as lenticules, which are intended for long-term *in vivo* use.

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1 Luck et al, U.S. Pat. No. 4,488,911, disclosed a method for preparing collagen in solution  
2 (CIS), wherein native collagen is extracted from animal tissue in dilute aqueous acid, followed by  
3 digestion with an enzyme such as pepsin, trypsin, or Pronase®, a trademark of American Hoechst  
4 Corporation, Somerville, NJ. The enzymatic digestion removes the telopeptide portions of the  
5 collagen molecules, providing "atelopeptide" collagen in solution. The atelopeptide CIS so pro-  
6 duced is substantially nonimmunogenic, and is also substantially non-crosslinked due to loss of the  
7 primary crosslinking regions. The CIS may then be precipitated by dialysis in a moderate shear  
8 environment to produce collagen fibers which resemble native collagen fibers. The precipitated,  
9 reconstituted fibers may additionally be crosslinked using a chemical agent (for example, aldehydes  
10 such as formaldehyde and glutaraldehyde), heat, or radiation. The resulting products are suitable  
11 for use in medical implants due to their biocompatibility and reduced immunogenicity.

12 Wallace et al, U.S. Pat. No. 4,424,208, disclosed an improved collagen formulation  
13 suitable for use in soft tissue augmentation. Wallace's formulation comprises reconstituted fibrillar  
14 atelopeptide collagen (for example, Zyderm® Collagen) in combination with particulate,  
15 crosslinked atelopeptide collagen dispersed in an aqueous medium. The addition of particulate  
16 crosslinked collagen improves the implant's persistence, or ability to resist shrinkage following  
17 implantation.

18 Smestad et al, U.S. Pat. No. 4,582,640, disclosed a glutaraldehyde crosslinked  
19 atelopeptide CIS preparation (GAX) suitable for use in medical implants. The collagen is cross-  
20 linked under conditions favoring interfiber bonding rather than intrafiber bonding, and provides a  
21 product with higher persistence than non-crosslinked atelopeptide collagen. Said product is com-  
22 mercially available from Collagen Corporation under the trademark Zyplast® Collagen Implant.

23 Nguyen et al, U.S. Pat. No. 4,642,117, disclosed a method for reducing the viscosity of  
24 atelopeptide CIS by mechanical shearing. Reconstituted collagen fibers are passed through a fine-  
25 mesh screen until viscosity is reduced to a practical level for injection.

26 Nathan et al, U.S. Pat. No. 4,563,350, disclosed osteoinductive bone repair compositions  
27 comprising an osteoinductive factor, at least 5% nonreconstituted (afibrillar) collagen, and the  
28 remainder reconstituted collagen and/or mineral powder (e.g., hydroxyapatite). CIS may be used  
29 for the nonreconstituted collagen, and Zyderm® Collagen Implant (ZCI) is preferred for the  
30 reconstituted collagen component. The material is implanted in bone defects or fractures to speed  
31 ingrowth of osteoclasts and promote new bone growth.

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1 Chu, U.S. Pat. No. 4,557,764, disclosed a "second nucleation" collagen precipitate which  
2 exhibits a desirable malleability and putty-like consistency. Collagen is provided in solution (e.g.,  
3 at 2-4 mg/mL), and a "first nucleation product" is precipitated by rapid titration and centrifugation.  
4 The remaining supernatant (containing the bulk of the original collagen) is then decanted and  
5 allowed to stand overnight. The precipitated second nucleation product is collected by centri-  
6 fugation.

7 Chu, U.S. Pat. No. 4,689,399, disclosed a collagen membrane preparation, which is  
8 prepared by compressing and drying a collagen gel. The resulting product has high tensile  
9 strength.

10 Silver et al., U.S. Pat. No. 4,703,108, disclosed the preparation of a sponge prepared by  
11 crosslinking insoluble collagen using dehydrothermal means or by using cyanamide. Berg et al.,  
12 U.S. Pat. No. 4,837,285, disclosed the preparation of collagen in bead form for soft tissue  
13 augmentation. Brodsky et al., U.S. Pat. No. 4,971,954, have disclosed a method of crosslinking  
14 collagen using ribose or other reducing sugars.

15 Miyata et al., Japanese patent application 63-256512, published August 17, 1992,  
16 discloses a composition comprised of atelopeptide collagen linked to a polyepoxy compound. The  
17 composition is injected into the body to obtain sustained skin-lifting effects.

18 J.A.M. Ramshaw et al, Anal Biochem (1984) 141:361-65, and PCT application  
19 WO87/04078, disclosed the precipitation of bovine collagen (types I, II, and III) from aqueous  
20 PEG solutions, where there is no binding between collagen and PEG.

21 Werner, U.S. Pat. No. 4,357,274, disclosed a method for improving the durability of  
22 sclero protein (e.g., brain meninges) by soaking the degreased tissue in hydrogen peroxide or  
23 polyethylene glycol for several hours prior to lyophilization. The resulting modified whole tissue  
24 exhibits increased persistence.

25 Hiroyoshi, U.S. Pat. No. 4,678,468, disclosed the preparation of polysiloxane polymers  
26 having an interpenetrating network of water-soluble polymer dispersed within. The water-soluble  
27 polymer may be a collagen derivative, and the polymer may additionally include heparin. The  
28 polymers are shaped into artificial blood vessel grafts, which are designed to prevent clotting.

29 Other patents disclose the use of collagen preparations incorporating bone fragments or  
30 minerals. For example, Miyata et al, U.S. Pat. No. 4,314,380, disclosed a bone implant prepared  
31 by baking animal bone segments, then soaking the baked segments in a solution of atelopeptide

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1 collagen. Deibig et al, U.S. Pat. No. 4,192,021, disclosed an implant material which comprises  
2 powdered calcium phosphate in a pasty formulation with a biodegradable polymer (which may be  
3 collagen). Commonly owned U.S. application Serial No. 06/855,004, filed April 22, 1986, now  
4 abandoned, disclosed a particularly effective bone repair material comprising autologous bone  
5 marrow, collagen, and particulate calcium phosphate in a solid, malleable formulation.

6 There are several references in the art to proteins modified by covalent conjugation to  
7 polymers to alter the solubility, antigenicity, and biological clearance of the protein. For example,  
8 U.S. Patent 4,261,973 disclosed the conjugation of several allergens to PEG or PPG  
9 (polypropylene glycol) to reduce the proteins' immunogenicity. U.S. Patent 4,301,144 disclosed  
10 the conjugation of hemoglobin with PEG and other polymers to increase the protein's oxygen-  
11 carrying capability. EP 098110 disclosed coupling an enzyme or interferon to a polyoxyethylene-  
12 polyoxypropylene (POE-POP) block polymer to increase the protein's half-life in serum. U.S.  
13 Patent 4,179,337 disclosed conjugating hydrophilic enzymes and insulin to PEG or PPG to reduce  
14 immunogenicity. Davis et al, Lancet (1981) 2:281-83, disclosed the enzyme uricase modified by  
15 conjugation with PEG to provide uric acid metabolism in serum having a long half-life and low  
16 immunogenicity. Nishida et al, J Pharm Pharmacol (1984) 36:354-55, disclosed PEG-uricase con-  
17 jugates administered orally to chickens, demonstrating decreased serum levels of uric acid. Inada  
18 et al, Biochem & Biophys Res Comm (1984) 122:845-50 disclosed lipoprotein lipase conjugated  
19 with PEG to render it soluble in organic solvents. Takahashi et al, Biochem & Biophys Res  
20 Comm (1984) 121:261-65, disclosed HRP conjugated with PEG to render the enzyme soluble in  
21 benzene. Abuchowski et al, Cancer Biochem Biophys (1984) 7:175-86, disclosed that enzymes  
22 such as asparaginase, catalase, uricase, arginase, trypsin, superoxide dismutase, adenosine  
23 deaminase, phenylalanine ammonia-lyase and the like conjugated with PEG exhibit longer half-  
24 lives in serum and decreased immunogenicity. However, these references are essentially con-  
25 cerned with modifying the solubility and biological characteristics of proteins administered in low  
26 concentrations in aqueous solution.

27 M. Chvapil et al, J Biomed Mater Res (1969) 3:315-32, disclosed a composition prepared  
28 from collagen sponge and a crosslinked ethylene glycol monomethacrylate-ethylene glycol  
29 dimethacrylate hydrogel. The collagen sponge was prepared by lyophilizing an aqueous mixture of  
30 bovine hide collagen and methylglyoxal, a tanning agent. The sponge-hydrogel composition was

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1 prepared by polymerizing ethylene glycol monomethacrylate and ethylene glycol dimethacrylate in  
2 the sponge.

3 A series of related patents disclose various types of collagen-containing materials. The  
4 patents are U.S. Patent 4,703,108, issued October 27, 1987; 4,861,714, issued August 29, 1989;  
5 4,863,856, issued September 5, 1989; 4,925,924, issued May 15, 1990; 4,970,298, issued  
6 November 13, 1990; and 4,997,753, issued March 5, 1991. All of these patents disclose collagen  
7 materials wherein type I, II, and III collagens are contacted with a crosslinking agent selected from  
8 the group consisting of a carbodiimide or a succinimidyl active ester. Various types of treatment  
9 may be carried out prior to or after crosslinking in order to form particular types of desired  
10 materials such as sponges and/or sheets.

11 In commonly owned U.S. Patent 5,162,430, issued November 10, 1992, we described  
12 conjugates whereby atelopeptide collagen is covalently crosslinked with synthetic hydrophilic  
13 polymers such as polyethylene glycol. Such conjugates are useful for a variety of applications,  
14 such as soft tissue augmentation and the formation of implants useful in bone repair. In U.S.  
15 application Serial No. 07/922,541, we disclose various activated forms of polyethylene glycol and  
16 various linkages which can be used to produce collagen-synthetic polymer conjugates having a  
17 range of physical and chemical properties. We now describe collagen-synthetic polymer  
18 conjugates formed using chemically modified forms of collagen, which impart to the conjugate  
19 specific properties, such as optical clarity and mechanical strength, making the conjugates  
20 particularly useful in devices for use in ophthalmic applications.

## 21 Summary of the Invention

22 Chemically modified collagen is covalently crosslinked with an activated synthetic  
23 hydrophilic polymer to form a conjugate. The conjugate can be formulated into compositions  
24 having sufficient clarity that the compositions can be used to produce various ophthalmic devices  
25 and materials. The synthetic hydrophilic polymer is preferably various forms of activated  
26 polymeric glycols, most preferably polyethylene glycol. The chemically modified collagen is  
27 preferably succinylated collagen, produced by reacting collagen with succinic anhydride, or  
28 methylated collagen, produced by reacting collagen with methanol.

29 Furthermore, different forms of chemically modified collagens (for example, methylated  
30 collagen and succinylated collagen) can be mixed in varying ratios prior to covalent crosslinking to

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1 produce transparent materials having a range of physical properties and different degrees of  
2 crosslinking.

3 The present invention provides materials comprised of the covalently bound chemically  
4 modified collagen-synthetic polymer conjugates which have good optical clarity (*i.e.*, greater than  
5 90% transmittance of light having a wavelength of 410 nm), mechanical strength, and moldability.

6 The chemically modified collagen-synthetic polymer conjugates of the present invention  
7 have optical properties such that the conjugates can be used to produce ophthalmic devices.

8 An advantage of the invention is that the chemically modified collagen-synthetic polymer  
9 conjugates can be used to produce products which are clear, strong, nonimmunogenic, and  
10 permeable to oxygen and other nutrients.

11 A feature of the invention is that the covalently bound chemically modified collagen-  
12 synthetic polymer conjugates are moldable and can be molded into an article which is sufficiently  
13 high in light transmittance that it may be considered clear, *i.e.*, of optical quality.

14 These and other features of the present invention will become apparent to those persons  
15 skilled in the art upon reading the details of the structure, synthesis, and usage of the conjugates  
16 and clear articles made therewith as more fully set forth below.

#### 17 Brief Description of the Drawings

18 Figure 1 shows differential scanning calorimetry (DSC) results for noncrosslinked  
19 succinylated collagen (Sample A) and succinylated collagen formulations containing 10, 20, 50,  
20 and 91 mg/ml S-PEG (Samples B, C, and D, respectively).

21 Figure 2 shows DSC results for methylated collagen formulations containing 2, 10, 30,  
22 and 72 mg/ml S-PEG (Samples E, F, G, and H, respectively).

23 Figure 3 shows DSC results for noncrosslinked methylated collagen.

#### 24 Detailed Description of Preferred Embodiments of the Invention

25 It must be noted that, as used in this specification and the appended claims, the singular  
26 forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise.



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1 Thus, for example, reference to "a conjugate" includes one or more conjugate molecules, reference  
2 to "an article" includes one or more different types of articles known to those skilled in the art and  
3 reference to "the collagen" includes mixtures of different types of collagens and so forth.

4 Unless defined otherwise, all technical and scientific terms used herein have the same  
5 meaning as commonly understood by one of ordinary skill in the art to which this invention  
6 belongs. Although any methods and materials similar or equivalent to those described herein may  
7 be useful in the practice or testing of the present invention, only the preferred methods and  
8 materials are described below; it is not intended that the invention be limited to these preferred  
9 embodiments, however. The invention is intended to have the scope defined by the attached  
10 claims.

11 All publications mentioned herein are incorporated herein by reference to describe and  
12 disclose the subject matter for which it is cited.

13 Specific terminology of particular importance to the description of the present invention is  
14 defined below.

#### 15 Definitions

16 The term "collagen" as used herein refers to all forms of collagen which can be used as  
17 starting materials, including those which have been recombinantly produced, extracted from  
18 naturally occurring sources, processed, or otherwise modified. Collagens may or may not be  
19 treated to remove the immunogenic telopeptide regions ("atelopeptide collagen"). Atelopeptide  
20 fibrillar collagen at approximately pH 7 is the preferred starting material to produce the chemically  
21 modified collagens which are covalently bound to synthetic hydrophilic polymers to produce the  
22 clear conjugates of the invention. Crosslinked collagen prepared using heat, radiation, or chemical  
23 agents such as glutaraldehyde is not preferred as a starting material for preparation of the  
24 compositions of the present invention. Collagen used in connection with the preferred  
25 embodiments of the invention is in a pharmaceutically pure form such that it can be incorporated  
26 into a human body for the intended purpose without generating any significant immune response.  
27 Collagen used to form a conjugate must form a conjugate which will allow the production of an  
28 ophthalmic device with acceptable optical properties.

29 The term "nonfibrillar collagen" refers to collagens in which the triple helical molecules do  
30 not aggregate to form fibers.

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1       The term "succinylated collagen" refers to collagen which has been reacted with succinic  
2 anhydride or other anhydrides, resulting in acylation of the free amino groups on the collagen  
3 molecule. A method for preparing succinylated collagen is disclosed in U.S. Patent No.  
4 4,164,559, which is incorporated herein by reference.

5       The term "methylated collagen" refers to collagen which has been reacted with methanol or  
6 another water-soluble aliphatic alcohol such as ethanol, resulting in esterification of the free  
7 carboxyl groups on the collagen molecule. A method for preparing methylated collagen is also  
8 disclosed in U.S. Patent No. 4,164,559.

9       Preferred synthetic polymers for use in the present invention are hydrophilic and are highly  
10 pure or are purified to a highly pure state such that the polymer is or is treated to become  
11 pharmaceutically pure so that it may be injected into a human patient. Most hydrophilic synthetic  
12 polymers can be rendered water-soluble by incorporating a sufficient number of oxygen (or less fre-  
13 quently nitrogen) atoms available for forming hydrogen bonds in aqueous solutions. Preferred  
14 synthetic polymers are hydrophilic, but not necessarily water-soluble. Hydrophilic synthetic  
15 polymers described in the preferred embodiments herein include activated forms of polyethylene  
16 glycol (PEG), polyoxyethylene, polymethylene glycol, polytrimethylene glycols, polyvinylpyrrol-  
17 idones, and derivatives thereof, with activated PEG being particularly preferred. The synthetic  
18 polymers can be linear or multiply branched, but are typically not substantially crosslinked. Other  
19 suitable hydrophilic synthetic polymers include, for example, polyoxyethylene-polyoxypropylene  
20 block polymers and copolymers. Polyoxyethylene-polyoxypropylene block polymers having an  
21 ethylenediamine nucleus (and thus having four ends) are commercially available and may be used  
22 in the practice of the invention. Naturally occurring polymers such as proteins, starch, cellulose,  
23 heparin, hyaluronic acid, derivatives thereof, and the like are expressly excluded from the scope of  
24 this definition. All suitable synthetic polymers will be nontoxic, noninflammatory, and  
25 nonimmunogenic when administered subcutaneously, and will preferably be essentially  
26 nondegradable *in vivo* over a period of at least several months. The hydrophilic synthetic polymer  
27 may increase the hydrophilicity of the conjugate, but does not render it water-soluble. The most  
28 preferred hydrophilic synthetic polymers include mono-, di-, and multifunctionally activated poly-  
29 meric glycols. Monofunctionally activated polymeric glycols have only one reactive hydroxy  
30 group, while difunctionally activated polymeric glycols typically have two reactive groups, one at  
31 each end of the polymer chain. Monofunctionally activated polymeric glycols preferably have an

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1 average molecular weight between about 100 and about 15,000, more preferably between about  
2 200 and about 8,000, and most preferably about 5,000. Difunctionally activated polymeric glycols  
3 preferably have an average molecular weight of between about 400 to about 40,000, most  
4 preferably about 3,000 to about 10,000. Multifunctionally activated polymeric glycols preferably  
5 have an average molecular weight between about 3,000 and 100,000.

6 Polymeric glycols can be rendered monofunctionally activated by forming an alkylene ether  
7 group at one end, for example. The alkylene ether group may be any suitable alkoxy radical  
8 having 1-6 carbon atoms, for example, methoxy, ethoxy, propoxy, 2-propoxy, butoxy, hexyloxy,  
9 and the like. Methoxy is presently preferred. Difunctionally activated polymeric glycols typically  
10 are prepared by constructing reactive hydroxy groups at the ends of the polymer. Multifunctionally  
11 activated synthetic polymers can be prepared using various techniques known in the art which  
12 provide active functional groups at various locations along the polymer. Multifunctionally  
13 activated synthetic polymers are capable of crosslinking the compositions of the invention, and  
14 may further be used to attach cytokines or growth factors to the nonfibrillar collagen-synthetic  
15 polymer conjugate.

16 The term "chemically conjugated" as used herein means attached through a covalent  
17 chemical bond. In the practice of the invention, a hydrophilic synthetic polymer and a chemically  
18 modified collagen may be covalently conjugated using a linking radical, so that the hydrophilic  
19 synthetic polymer and chemically modified collagen are each bound to the radical, but not directly  
20 to each other. The term "collagen-polymer" refers to collagen chemically conjugated to a synthetic  
21 hydrophilic polymer, within the meaning of this invention. For example, "PEG-collagen" or  
22 "collagen-PEG" denotes a composition of the invention wherein collagen is covalently conjugated  
23 to PEG. "Collagen-dPEG" is a subset of collagen-PEG and refers to collagen covalently  
24 conjugated to a difunctionally activated PEG, wherein the collagen molecules are crosslinked by  
25 means of the two functional ends of each PEG molecule. "Crosslinked collagen" refers to collagen  
26 in which collagen molecules are linked by covalent bonds with multifunctionally activated (includ-  
27 ing the difunctionally activated) synthetic hydrophilic polymers. The activated hydrophilic  
28 synthetic polymer may be "covalently conjugated" to the nonfibrillar collagen by means of a  
29 number of different types of chemical linkages. For example, the conjugation can be via an ester  
30 or a urethane linkage, but is more preferably by means of an ether linkage. An ether linkage is

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1 preferred in that it can be formed without the use of toxic chemicals and is not readily susceptible to  
2 hydrolysis *in vivo*.

3 Those of ordinary skill in the art will appreciate that synthetic polymers such as poly-  
4 ethylene glycol cannot be prepared practically to have exact molecular weights, and that the term  
5 "molecular weight" as used herein refers to the weight average molecular weight of a number of  
6 molecules in any given sample, as commonly used in the art. Thus, a sample of PEG 2,000 might  
7 contain a statistical mixture of polymer molecules ranging in weight from, for example, 1,500 to  
8 2,500, with one molecule differing slightly from the next over a range. Specification of a range of  
9 molecular weight indicates that the average molecular weight may be any value between the limits  
10 specified, and may include molecules outside those limits. Thus, a molecular weight range of  
11 about 800 to about 20,000 indicates an average molecular weight of at least about 800, ranging up  
12 to about 20,000.

13 The term "available lysine residue" as used herein refers to lysine side chains exposed on  
14 the outer surface of collagen molecules, which have free amino groups capable of reacting with  
15 activated polymeric glycols. The number of available lysine residues may be determined by reac-  
16 tion with sodium 2,4,6-trinitrobenzenesulfonate (TNBS).

17 The terms "treat" and "treatment" as used herein refer to replacement, augmentation, repair,  
18 prevention, or alleviation of defects related to soft tissue and, in particular, ocular tissue. Addition-  
19 ally, "treat" and "treatment" also refer to the prevention, maintenance, or alleviation of disorders or  
20 disease using a biologically active protein coupled to or mixed with the conjugates or articles of the  
21 invention. Treatment of ocular tissue includes the augmentation of corneal tissue with an article  
22 having suitable ocular properties to effect refractive correction.

23 The terms "cytokine" and "growth factor" are used to describe biologically active molecules  
24 and active peptides (which may be either naturally occurring or synthetic) which aid in healing or  
25 regrowth of normal tissue and, in particular, ocular tissue. The function of cytokines and growth  
26 factors is two-fold: 1) they can incite local cells to produce new tissue, or 2) they can attract cells  
27 to the site in need of correction. As such, cytokines or growth factors serve to encourage  
28 "biological anchoring" of any article or implant within the host tissue. As previously described,  
29 the cytokines and growth factors can either be admixed with the collagen-synthetic polymer  
30 conjugate or chemically coupled to the conjugate. For example, one may incorporate cytokines  
31 such as interferons (IFN), tumor necrosis factors (TNF), interleukins, colony stimulating factors

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1 (CSFs), or growth factors such as osteogenic factor extract (OFE), epidermal growth factor  
2 (EGF), transforming growth factor (TGF) alpha, TGF- $\beta$  (including any combination of TGF- $\beta$ s),  
3 TGF- $\beta$ 1, TGF- $\beta$ 2, platelet derived growth factor (PDGF-AA, PDGF-AB, PDGF-BB), acidic  
4 fibroblast growth factor (FGF), basic FGF, connective tissue activating peptides (CTAP),  
5  $\beta$ -thromboglobulin, insulin-like growth factors, erythropoietin (EPO), nerve growth factor (NGF),  
6 and the like. Incorporation of cytokines, growth factors, and appropriate combinations of  
7 cytokines and growth factors can facilitate the regrowth and remodeling of a device or implant into  
8 normal tissue. Furthermore, one may chemically link the cytokines or growth factors to the  
9 collagen-polymer conjugate by employing a suitable amount of multifunctional polymer molecules  
10 during synthesis. The cytokines or growth factors may then be attached to the activated sites on  
11 the synthetic polymer by the same method used to attach PEG to collagen, or by another suitable  
12 method. By tethering cytokines or growth factors to the implant, the amount of cytokine or growth  
13 factor required to achieve a desired effect is substantially reduced. Devices incorporated with  
14 cytokines or growth factors may serve as effective controlled release drug delivery means. By  
15 varying the chemical linkage between the collagen and the synthetic polymer, it is possible to vary  
16 the effect with respect to the release of the cytokine. For example, when an "ester" linkage is used,  
17 the linkage is more easily broken under physiological conditions, allowing for sustained release of  
18 the growth factor from the matrix. However, when an "ether" linkage is used, the bonds are not  
19 easily broken and the cytokine or growth factor will remain in place for longer periods of time with  
20 its active sites exposed, providing a biological effect on the natural substrate for the active site of  
21 the protein. It is possible to include a mixture of conjugates with different linkages so as to obtain  
22 variations in the effect with respect to the release of the cytokine, *i.e.*, the sustained release or  
23 controlled release effect can be modified to obtain the desired rate of release.

24 The term "effective amount" refers to the amount of composition required in order to obtain  
25 the effect desired. Thus, a "tissue growth-promoting amount" of a composition containing a  
26 cytokine refers to the amount of cytokine needed in order to stimulate tissue growth to a detectable  
27 degree. Tissue, in this context, includes any tissue, although ocular tissue is of particular interest  
28 in connection with the invention. The actual amount which is determined to be an effective amount  
29 will vary depending on factors such as the size, condition, sex, and age of the patient, and can be  
30 more readily determined by the caregiver.

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1 The term "sufficient amount" as used herein is applied to the amount of carrier used in  
2 combination with the collagen-polymer conjugates of the invention. A sufficient amount is that  
3 amount which, when mixed with the conjugate, renders it in the physical form desired, for  
4 example, the conjugate may include a sufficient amount of liquid carrier for creating a malleable  
5 composition used to create a corneal implant having a gel-like consistency and texture similar to a  
6 soft contact lens. The flowable formulations generally include an amount of a carrier sufficient to  
7 render the composition smoothly flowable at room temperature. The amount of the carrier can be  
8 varied and adjusted depending on the particular conjugate used and the end result desired. Such  
9 adjustments will be apparent to those skilled in the art reading this disclosure.

10 The terms "implant" and "solid implant" refer to any solid object which is designed for  
11 insertion and use within the body, and includes ocular implants such as ophthalmic shields and  
12 corneal lenticules.

13 The term "*in situ*" as used herein means at the site of administration. For example,  
14 ophthalmic devices can be placed in the eye of a patient and drugs such as a cytokine dispersed  
15 therein will be released *in situ*.

16 The term "dehydrated" means that the material is air-dried or lyophilized to remove  
17 substantially all unbound water.

18 The term "aqueous mixture" of collagen includes liquid solutions, suspensions,  
19 dispersions, colloids, and the like containing collagen and water.

20 The term "clear" as used herein refers to an article which transmits at least 90% of the  
21 visible light directed at it at a thickness of 1 mm.

## 22 General Method

23 In order to produce the ophthalmic devices of the invention, it is necessary to produce  
24 chemically modified collagen-synthetic polymer conjugates of the kind previously described, from  
25 which the devices can be fabricated. In order to produce such conjugates, it is necessary to  
26 covalently bind chemically modified nonfibrillar collagen to an activated synthetic hydrophilic  
27 polymer such as, for example, an activated polymeric glycol. In accordance with one preferred  
28 method, (a) collagen is chemically modified by succinylation, methylation, or deamidation to  
29 produce collagen which is substantially nonfibrillar at pH 7, (b) the hydrophilic synthetic polymer  
30 is activated, and (c) the activated synthetic polymer is allowed to react with the chemically

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1 modified collagen, resulting in the formation of covalent crosslinks between the synthetic polymer  
2 and the collagen.

3 Chemical Modification of Fibrillar Collagen to Produce Nonfibrillar Collagen

4 Suitable collagens for use as starting materials to produce the chemically modified collagen-  
5 synthetic polymer conjugates of the invention include all types of pharmaceutically useful  
6 collagens, preferably types I, II, and III. Collagens may be soluble (for example, commercially  
7 available Vitrogen® 100 collagen-in-solution, a trademark of Celtrix Pharmaceuticals, Santa Clara,  
8 California), and may or may not have the telopeptide regions. The collagen may be reconstituted  
9 fibrillar atelopeptide collagen, for example, Zyderm® Collagen Implant (ZCI), a trademark of  
10 Collagen Corporation, Palo Alto, California, or atelopeptide collagen in solution (CIS). Various  
11 forms of collagen are available commercially, or may be prepared by the processes described in,  
12 for example, U.S. Pat. Nos. 3,949,073; 4,488,911; 4,424,208; 4,582,640; 4,642,117;  
13 4,557,764; and 4,689,399, all incorporated herein by reference. Fibrillar atelopeptide  
14 reconstituted collagen is the presently preferred starting material.

15 Unmodified collagen, which is in fibrillar form at pH 7, is an opaque whitish viscous fluid  
16 unsuitable for use in the ophthalmic devices of the invention. Unmodified collagen has a net  
17 neutral charge at pH 7. Fiber formation occurs as a result of ionic interactions between opposite  
18 charges on various parts of the collagen molecule. Ionic interactions between collagen molecules  
19 causes the linear molecules to assemble into thick fibers.

20 As disclosed in U.S. Pat. No. 4,164,559, succinylated collagen is produced by reacting  
21 fibrillar collagen with succinic anhydride. Reaction of fibrillar collagen with succinic anhydride  
22 results in acylation of the free amino groups on the lysine residues of the collagen molecule. Other  
23 acylating agents that can be used include other anhydrides, such as acetic anhydride. Acylation of  
24 the amino groups results in the succinylated collagen molecules having a net negative charge at pH  
25 7, preventing the ionic interactions which result in fiber formation. However, in an acidic  
26 environment, the negative charges are balanced by the free H<sup>+</sup> ions in the solution, resulting in a  
27 net neutral charge for the molecule and allowing fiber formation to occur. Succinylated collagen is  
28 in substantially nonfibrillar form above pH 5.5.

29 It must be pointed out that complete succinylation will result in acylation of all of the free  
30 amino groups on the lysine residues, which would leave no free amino groups available for

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1 crosslinking with the synthetic hydrophilic polymer. The collagen should therefore be  
2 incompletely succinylated, leaving at least a minimum percentage of amino groups available for  
3 covalent crosslinking with the activated synthetic polymer. The degree of succinylation can be  
4 controlled by carefully controlling the relative amounts of succinic acid reacted with the collagen.  
5 The preferred degree of succinylation is in the range of 25 to 50%, meaning that 25 to 50% of the  
6 available amino groups on the lysine residues of the collagen molecule are succinylated. Collagen  
7 succinylated to such a degree will possess an adequate number of sites for covalent crosslinking  
8 with the activated synthetic polymer and still result in a crosslinked material having the desired  
9 degree of clarity. Collagen succinylated at a level significantly below 25% may not be clear or  
10 stable. The percentage of free lysine residues available for crosslinking with the activated synthetic  
11 hydrophilic polymer can be determined by reaction with TNBS (e.g., 75% free lysine residues  
12 indicates that an average of approximately 25% of amino groups on the lysine residues of the  
13 collagen molecules in the sample are succinylated).

14 In one preferred method, collagen-in-solution (pH 2) is adjusted to pH 9 to produce  
15 fibrillar collagen, which is then reacted with succinic anhydride to produce succinylated collagen.  
16 The pH of the resulting succinylated collagen is subsequently adjusted to 5 or less, causing the  
17 succinylated collagen to precipitate, i.e., form fibers. The precipitated succinylated collagen is then  
18 concentrated by centrifugation. At this point, any unreacted fibrillar collagen is in solution  
19 (because of the low pH) and is discarded with the supernatant, leaving only precipitated  
20 succinylated collagen. The pH of the concentrated succinylated collagen is adjusted to 7 to cause  
21 the collagen fibers to disassemble, resulting in nonfibrillar collagen. The resulting succinylated  
22 collagen is then adjusted to the desired collagen concentration by adding water.

23 As disclosed in U.S. Pat. No. 4,164,559, methylated collagen is prepared by reacting  
24 fibrillar collagen with methanol. Reaction of fibrillar collagen with methanol (or other water-  
25 soluble aliphatic alcohol such as ethanol) results in esterification of free carboxyl groups on the  
26 collagen. Esterification of the carboxyl groups results in the methylated collagen molecules having  
27 a net positive charge at pH 7, preventing the ionic interactions which result in fiber formation.  
28 However, in a basic environment, the positive charges are balanced by the free OH<sup>-</sup> ions in the  
29 solution, resulting in a net neutral charge for the molecule and allowing fiber formation to occur.  
30 Methylated collagen is in substantially nonfibrillar form below pH 9.



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1           Reaction with methanol does not affect the free amino groups on the collagen; therefore, it  
2 is possible to use collagen methylated to a level as high as 100% to form the conjugates of the  
3 invention. The degree of methylation can be controlled by limiting the reaction time of the  
4 methanol with the collagen.

5           In one preferred method, fibrillar collagen, such as Zyderm® I (35 mg/ml) or Zyderm II  
6 (65 mg/ml) Collagen (both available from Collagen Corporation, Palo Alto, CA), is lyophilized to  
7 produce freeze-dried collagen, which is subsequently chopped into small pieces. The chopped,  
8 freeze-dried fibrillar collagen is reacted with anhydrous acidic methanol and incubated at room  
9 temperature for 2 to 7 days to produce methylated collagen. The excess methanol is subsequently

10 evaporated off and the resulting methylated collagen lyophilized and dialyzed. The collagen  
11 concentration of the methylated collagen may be adjusted at this point.

12           Succinylation or methylation of the collagen molecule provides collagen which is  
13 substantially nonfibrillar and, therefore, clear at physiological pH, in contrast to unmodified  
14 collagen, which is fibrillar and milky white at physiological pH. However, as described  
15 previously, the chemically modified collagens themselves do not have the mechanical strength or  
16 physical properties required to form ophthalmic devices intended for long-term use in the eye.  
17 Further, the chemically modified collagens are inherently more unstable than unmodified fibrillar  
18 collagen due to the stability imparted by the fibrillar structure. However, reaction of the chemically  
19 modified collagens with activated synthetic hydrophilic polymers provides stable, covalently  
20 crosslinked conjugates having the mechanical strength and physical characteristics required for use  
21 in the ophthalmic devices of the invention.

#### 22           Activation of Polyethylene Glycol (PEG)

23           The first step in forming the conjugates of the invention generally involves  
24 functionalization, or activation, of the synthetic hydrophilic polymer. Although different synthetic  
25 hydrophilic synthetic polymers can be used in connection with forming the conjugate, the polymer  
26 must be biocompatible, hydrophilic, but relatively insoluble in water, and is preferably one or more  
27 forms of derivatized polyethylene glycol (PEG), due to its known biocompatibility. Various forms  
28 of derivatized PEG are extensively used in the modification of biologically active molecules  
29 because PEG can be formulated to have a wide range of solubilities and because it lacks toxicity,  
30

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1 antigenicity, immunogenicity, and does not typically interfere with the enzymatic activities and/or  
2 conformations of peptides. Furthermore, PEG is generally non-biodegradable and is easily  
3 excreted from most living organisms including humans.

4 Various functionalized polyethylene glycols have been used effectively in fields such as  
5 protein modification (see Abuchowski et al., Enzymes as Drugs, John Wiley & Sons: New York,  
6 NY (1981) pp. 367-383; and Dreborg et al., Crit. Rev. Therap. Drug Carrier Syst. (1990) 6:315 ),  
7 peptide chemistry (see Mutter et al., The Peptides, Academic: New York, NY 2:285-332; and  
8 Zalipsky et al., Int. J. Peptide Protein Res. (1987) 30:740), and the synthesis of polymeric drugs  
9 (see Zalipsky et al., Eur. Polym. J. (1983) 19:1177; and Ouchi et al., J. Macromol. Sci. -Chem.  
10 (1987) A24:1011). Various types of conjugates formed by the binding of activated  
11 (functionalized) polyethylene glycol with specific pharmaceutically active proteins have been  
12 disclosed and found to have useful medical applications in part due to the stability of such  
13 conjugates with respect to proteolytic digestion, reduced immunogenicity, and longer half-lives  
14 within living organisms.

15 One form of polyethylene glycol which has been found to be particularly useful is  
16 monomethoxy-polyethylene glycol (mPEG), which can be activated by the addition of a compound  
17 such as cyanuric chloride, then coupled to a protein (see Abuchowski et al., J. Biol. Chem. (1977)  
18 252:3578). Although such methods of activating polyethylene glycol can be used in connection  
19 with the present invention, they are not preferred in that the cyanuric chloride is relatively toxic and  
20 must be completely removed from any resulting product in order to provide a pharmaceutically  
21 acceptable composition.

22 Activated forms of PEG, including activated forms of mPEG, can be made from reactants  
23 which can be purchased commercially. One form of activated PEG which has been found to be  
24 particularly useful in connection with the present invention is mPEG-succinate-N-  
25 hydroxysuccinimide ester (SS-PEG) (see Abuchowski et al., Cancer Biochem. Biophys. (1984)  
26 7:175). Activated forms of PEG such as SS-PEG react with the proteins under relatively mild  
27 conditions and produce conjugates without destroying the specific biological activity and specificity  
28 of the protein attached to the PEG. However, when such activated PEGs are reacted with proteins,  
29 they react and form linkages by means of ester bonds. Although ester linkages can be used in  
30 connection with the present invention, they are not particularly preferred in that they undergo  
31 hydrolysis when subjected to physiological conditions over extended periods of time (see Dreborg

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1 et al., Crit. Rev. Therap. Drug Carrier Syst. (1990) 6:315; and Ulbrich et al., J. Makromol.  
2 Chem. (1986) 187:1131).

3 It is possible to link PEG to proteins via urethane linkages, thereby providing a more stable  
4 attachment which is more resistant to hydrolytic digestion than the ester linkages (see Zalipsky et  
5 al., Polymeric Drug and Drug Delivery Systems, Chapter 10, "Succinimidyl Carbonates of  
6 Polyethylene Glycol" (1991)). The stability of urethane linkages has been demonstrated under  
7 physiological conditions (see Veronese et al., Appl. Biochem. Biotechnol. (1985) 11:141; and  
8 Larwood et al., J. Labelled Compounds Radiopharm. (1984) 21:603). Another means of attaching  
9 the PEG to a protein can be by means of a carbamate linkage (see Beauchamp et al., Anal.  
10 Biochem. (1983) 131:25; and Berger et al., Blood (1988) 71:1641). The carbamate linkage is  
11 created by the use of carbonyldiimidazole-activated PEG. Although such linkages have  
12 advantages, the reactions are relatively slow and may take 2 to 3 days to complete.

13 The various means of activating PEG described above and publications cited in connection  
14 with the activation means are described in connection with linking the PEG to specific biologically  
15 active proteins and not inert, biologically inactive, natural polymers such as collagen. (See  
16 Polymeric Drug and Drug Delivery Systems, Chapter 10, "Succinimidyl Carbonates of  
17 Polyethylene Glycol" (1991).) However, the present invention now discloses that such activated  
18 PEG compounds can be used in preparation of covalently crosslinked conjugates of various  
19 chemically modified collagens to provide a conjugate having sufficient optical characteristics, such  
20 as optical clarity, that the conjugate can be used to form various compositions for use in ophthalmic  
21 and other medical applications.

## 22 Specific Forms of Activated PEG

23 For use in the present invention, polyethylene glycol is modified in order to provide  
24 activated groups on one or, preferably, two or more sites along the length of the PEG molecule, so  
25 that covalent binding can occur between the PEG and the free amino groups on a chemically  
26 modified collagen molecule. Some specific activated forms of PEG are shown structurally below,  
27 as are generalized reaction products obtained by reacting activated forms of PEG with chemically  
28 modified collagens. In Formulas 1-7, the term COLLAGEN is used to represent various forms of  
29 chemically modified collagens, such as succinylated collagen, methylated collagen, and deamidated

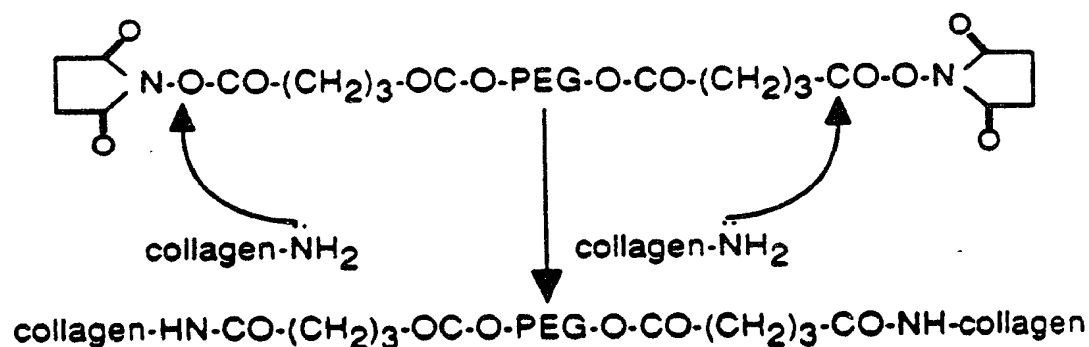
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1 collagen, for example. The term PEG is used to represent polymers having the repeating structure  
 2  $(\text{CH}_2 \text{CH}_2 \text{OCH}_2 \text{CH}_2)_n$ .

3 The first activated PEG is difunctionally activated PEG succinimidyl glutarate, referred to  
 4 herein as (SG-PEG). The structural formula of this molecule and the reaction product obtained by  
 5 reacting it with a chemically modified collagen are shown in Formula 1.

6 SG-PEG: Difunctionally Activated PEG Succinimidyl Glutarate

S-PEG: Difunctional PEG Succinimidyl Glutarate



7 FORMULA 1

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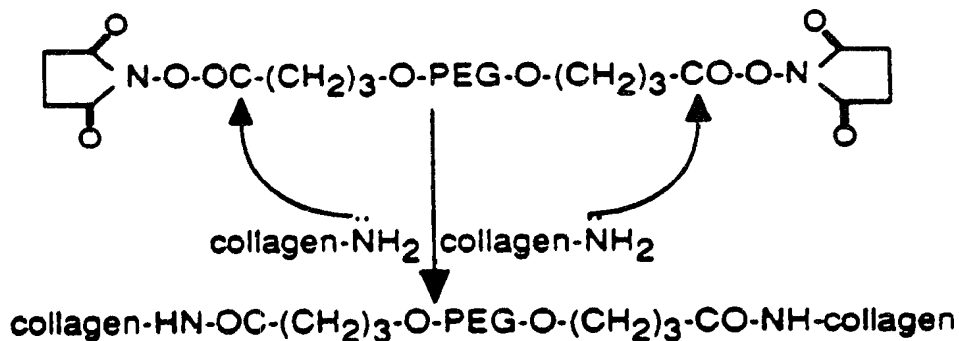
Another difunctionally activated form of PEG is referred to as PEG succinimidyl (S-PEG).

The structural formula for this compound and the reaction product obtained by reacting it with a chemically modified collagen such as methylated collagen is shown in Formula 2. In any general structural formula for the compounds, the subscript 3 is replaced with an "n". In the embodiment shown in Formula 1, n=3, in that there are three repeating CH<sub>2</sub> groups on either side of the PEG.

The structure in Formula 2 results in a conjugate which includes an "ether" linkage which is not subject to hydrolysis. This is distinct from the conjugate shown in Formula 1, wherein an ester linkage is provided. The ester linkage is subject to hydrolysis under physiological conditions.

S-PEG, n=3: Difunctionally Activated PEG Succinimidyl

S-PEG, n=3: Difunctional PEG Succinimidyl



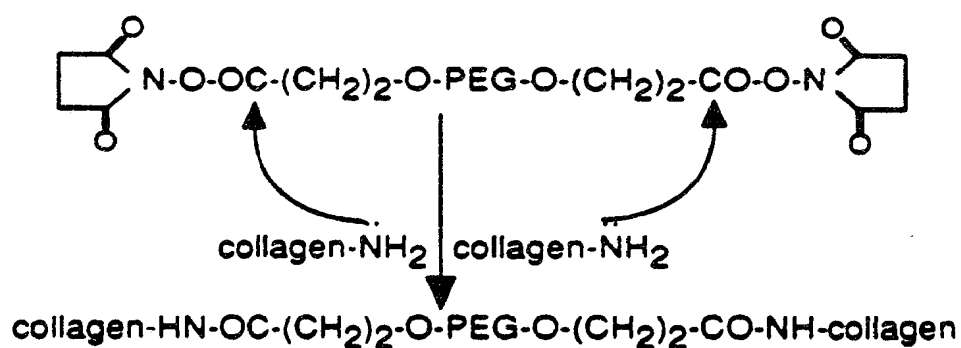
FORMULA 2

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- 1 Yet another difunctionally activated form of polyethylene glycol, wherein  $n=2$ , is shown in  
 2 Formula 3, as is the conjugate formed by reacting the activated PEG with a chemically modified  
 3 collagen.

4 S-PEG,  $n=2$ : Difunctionally Activated PEG Succinimidyl

S-PEG,  $n=2$ : Difunctional PEG Succinimidyl



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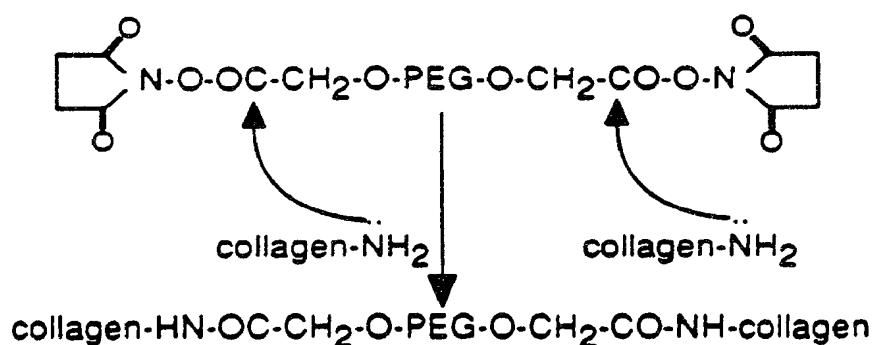
FORMULA 3

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- 1 Another preferred embodiment of the invention similar to the compounds of Formulas 2  
 2 and 3 is provided when  $n=1$ . The structural formula and resulting chemically modified collagen-  
 3 synthetic polymer conjugate are shown in Formula 4. It is noted that this conjugate includes both  
 4 an ether and a peptide linkage. These linkages are stable under physiological conditions.

5 S-PEG,  $n=1$ : Difunctionally Activated PEG Succinimidyl

S-PEG,  $n=1$ : Difunctional PEG Succinimidyl



6

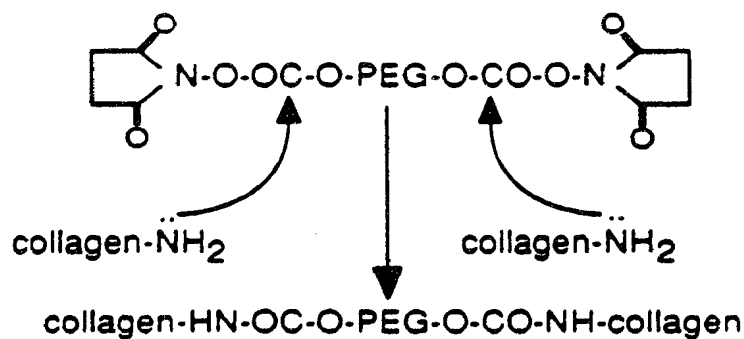
FORMULA 4

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1 Yet another difunctionally activated form of PEG is provided when  $n=0$ . This compound  
 2 is referred to as PEG succinimidyl carbonate (SC-PEG). The structural formula of this compound  
 3 and the conjugate formed by reacting SC-PEG with a chemically modified collagen is shown in  
 4 Formula 5.

5 SC-PEG,  $n=0$ ; Difunctionally Activated PEG Succinimidyl Carbonate

SC-PEG,  $n=0$ ; Difunctional PEG Succinimidyl Carbonate



6

FORMULA 5

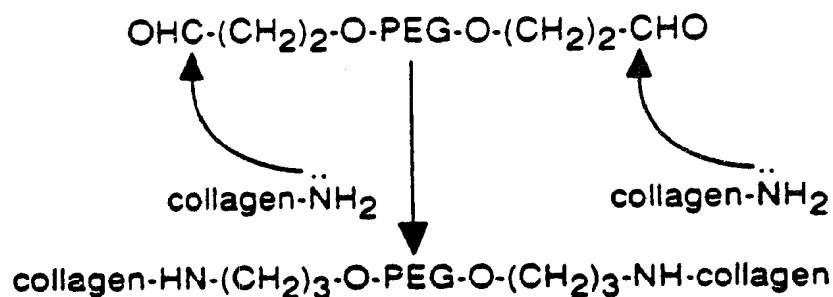


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1 All of the activated polyethylene glycol derivatives depicted in Formulas 1-5 involve the  
 2 inclusion of the succinimidyl group. However, different activating groups can be attached at sites  
 3 along the length of the PEG molecule. For example, PEG can be derivatized to form difunctionally  
 4 activated PEG propion aldehyde (A-PEG), which is shown in Formula 6, as is the conjugate  
 5 formed by the reaction of A-PEG with a chemically modified collagen. The linkage shown in  
 6 Formula 6 is referred to as a  $-(CH_2)_n-NH-$  linkage, where  $n=1-10$ .

7 A-PEG: Difunctionally Activated PEG Propion Aldehyde

A-PEG: Difunctional PEG Propion Aldehyde



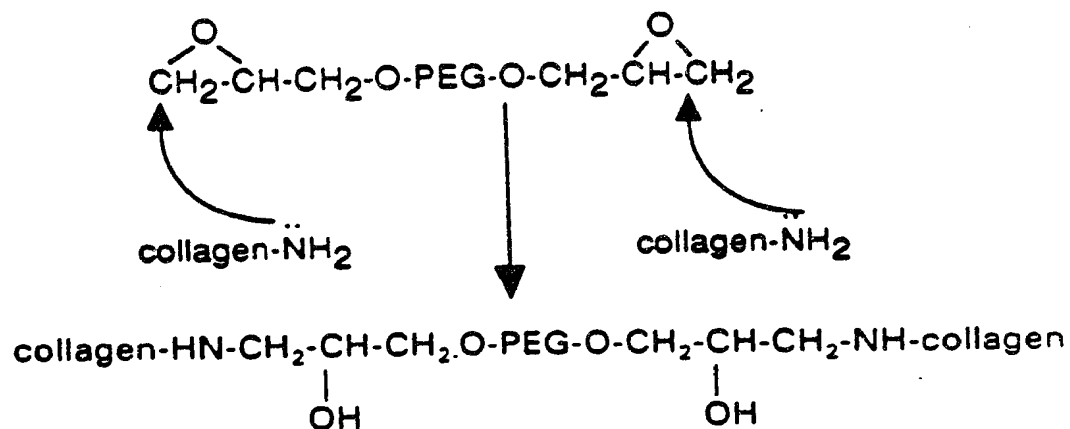
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FORMULA 6

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- 1 Yet another form of activated polyethylene glycol is difunctionally activated PEG glycidyl  
 2 ether (E-PEG), which is shown in Formula 7, as is the conjugate formed by reacting such with a  
 3 chemically modified collagen.

4 E-PEG: Difunctionally Activated PEG Glycidyl Ether



5 FORMULA 7

- 6 Many of the activated forms of polyethylene glycol described above are now available  
 7 commercially from Shearwater Polymers, Huntsville, Alabama.

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1 Crosslinking of Chemically Modified Collagen with PEG

2 Chemically modified collagens which have free amino groups can be covalently crosslinked  
3 by mixing with an appropriate amount of a multifunctionally (preferably difunctionally) activated  
4 synthetic hydrophilic polymer such as an activated polyethylene glycol. Chemically modified  
5 collagens retain their optical clarity after crosslinking with PEG to form clear gels having improved  
6 mechanical strength.

7 Covalently bound chemically modified collagen-synthetic polymer conjugates are formed  
8 within minutes of combining the chemically modified collagen with the functionally activated  
9 polymer. The chemically modified collagen can be mixed with the activated polymer using  
10 syringe-to-syringe mixing. Alternatively, the chemically modified collagen can be extruded into a  
11 solution of the activated polymer; crosslinking will occur as the polymer diffuses into the collagen.  
12 For crosslinking to occur between the synthetic polymer and the collagen, the pH of the reaction  
13 mixture must be maintained between approximately 6 and approximately 9.

14 The rate of conjugate formation and the characteristics of the resulting conjugate can be  
15 varied by varying the type of activated polymeric glycol used and/or the molecular weight and  
16 concentration of the polymeric glycol. In general, the use of polymeric glycol species (such as  
17 S-PEG) which result in ether or urethane linkages lead to the creation of more stable conjugates  
18 than those which result in the readily hydrolyzed ester linkages. Conjugates containing ether  
19 linkages are generally preferred for use in ophthalmic devices and materials intended for long-term  
20 use *in vivo*, such as lenticules. However, in certain situations, such as drug delivery applications,  
21 it is desirable to include the weaker ester linkages: the linkages are gradually broken by hydrolysis  
22 under physiological conditions, breaking apart the matrix and releasing the pharmaceutically active  
23 component held therein. Articles such as corneal shields where separation of the drug from the  
24 collagen-synthetic polymer matrix is required would generally incorporate weaker linkages such as  
25 ester linkages. Different species of polymeric glycols can be mixed and used in the same drug  
26 delivery composition, resulting in a varied rate of matrix degradation and, hence, drug release.

27 The specific concentration of activated polymeric glycol used will vary depending on the  
28 kind of polymeric glycol and the type of chemically modified collagen used, but is typically within  
29 the range of between about 1 to about 400 milligrams of polymeric glycol per milliliter of  
30 chemically modified collagen-synthetic polymer composition. For example, the preferred  
31 polymeric glycol concentration for preparing the optically clear compositions of the invention is in

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1 the range of from about 5 to about 70 milligrams of PEG per milliliter of succinylated collagen-  
2 synthetic polymer composition, and between about 1 to about 50 milligrams of PEG per milliliter  
3 of methylated collagen-synthetic polymer composition. The collagen concentration of the  
4 chemically modified collagen-synthetic polymer compositions is typically within the range of about  
5 5 to about 120 mg/ml, depending on the physical properties required for the desired end use of the  
6 composition.

7 In one preferred embodiment, for example, 10 mg of a difunctionally activated  
8 polyethylene glycol is dissolved in 0.1 ml of PBS to make a crosslinker solution. The 0.1 ml of  
9 crosslinker solution is then mixed with, for example, 0.9 ml of succinylated collagen having a  
10 collagen concentration of 30 mg/ml. The total volume of the succinylated collagen-synthetic  
11 polymer composition is now 1.0 ml. The final composition contains a total of 10 mg of  
12 difunctionally activated S-PEG and 27 mg of succinylated collagen (0.9 ml x 30 mg/ml collagen  
13 concentration).

14 The amount of activated polymeric glycol used and, hence, the degree of crosslinking will  
15 also vary depending on the desired end use of the material produced. For example, conjugates for  
16 use as vitreous gel replacements will need to be in a more fluid form, and thus require lighter  
17 crosslinking, than conjugates for use in solid implants, such as lenticules, which require formation  
18 of a firm, yet elastic gel. Presently preferred PEGs include difunctionally activated PEG  
19 succinimidyls (S-PEG), wherein  $n=0, 1, 2, 3$ , or 4, as depicted in Formulas 2-4. The physical  
20 form (*e.g.*, solid implant, viscous gel) of the resulting chemically modified collagen-synthetic  
21 polymer compositions made using a difunctionally activated S-PEG will depend upon the relative  
22 amount of S-PEG used in the composition.

23 Multifunctionally activated polymeric glycols can be used to crosslink more than one  
24 chemically modified species of collagen. The resulting composite material will have different  
25 physical and chemical properties than conjugates involving only one species of chemically  
26 modified collagen.

27 Multifunctionally activated synthetic polymers may further be used to crosslink chemically  
28 modified collagen to other proteins such as growth factors or cytokines to produce compositions  
29 particularly suited for use in wound healing and immune modulation. Such tethering of cytokines  
30 or growth factors to chemically modified collagen provides an effective sustained release drug

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1 delivery system. Further, tethered cytokines can be used to anchor the composition in place,  
2 providing increased useful lifetime for the implant.

3 Compositions of the invention containing biologically active cytokines or growth factors  
4 such as TGF- $\beta$  are prepared by admixing an appropriate amount of the cytokine or growth factor  
5 into the composition, or by incorporating the cytokine or growth factor into the chemically  
6 modified collagen prior to reaction with an activated polymeric glycol. Preferably, the cytokine or  
7 growth factor is first reacted with a molar excess of a multifunctionally activated polymeric glycol  
8 in a dilute solution for three to four minutes. The cytokine or growth factor is preferably provided  
9 at a concentration of about 1  $\mu\text{g/mL}$  to about 5  $\text{mg/mL}$ , while the activated polymer is preferably  
10 added to a final concentration providing a thirty- to fifty-fold molar excess. The conjugated  
11 biologically active factor-synthetic polymer is then added to an aqueous solution of chemically  
12 modified collagen (preferably having a concentration within the range of about 1 to about 60  
13  $\text{mg/mL}$ ) at neutral pH (approximately 7 - 8) and allowed to react further to form biologically active  
14 factor-synthetic polymer-chemically modified collagen conjugates. The resulting composition is  
15 allowed to stand overnight at ambient temperature. The pellet is collected by centrifugation and  
16 washed with PBS, using vigorous vortexing to remove unbound factor. Methods for conjugating  
17 growth factors and cytokines to the collagen-polymer conjugate are further described in U.S.  
18 Patent 5,162,430, which is incorporated herein by reference.

#### 19 Use and Administration

20 Compositions comprising the chemically modified collagen-synthetic polymer conjugates  
21 of the present invention are generally used to form various ophthalmic devices, but can be used in  
22 the formation of a wide variety of materials used in connection with medical treatment. The  
23 conjugate formulations of the invention are particularly advantageous in that they provide materials  
24 and devices which are relatively transparent to visible light. Accordingly, the advantages of the  
25 present invention become more apparent when the invention is used to form a device for which  
26 optical clarity is a requirement.

27 Due to their optical clarity (greater than 90% transmittance of light at a wavelength of about  
28 410 nm for an article having a thickness of 1 mm) and moldability, the conjugates are ideal for use  
29 in various materials and devices to be incorporated in the eye. For instance, they can be used as  
30 vitreous humor replacements, as corneal shields to deliver drugs to the eye, or as artificial corneal

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1 implants. As such, the conjugates can be used to deliver various drugs and therapeutic agents,  
2 such as TGF- $\beta$ , antiglaucoma agents, steroids, antibiotics, to both the anterior and posterior  
3 chambers of the eye, depending on the desired therapeutic effect. As described above, the  
4 therapeutic agents may either be admixed with the chemically modified collagen-synthetic polymer  
5 conjugates or covalently bound to the conjugates.

6 Alternatively, the chemically modified collagen-synthetic polymer conjugates can be used  
7 as starting materials in the procedure for synthetic epikeratoplasty. As such, the conjugates are  
8 shaped into preformed lenticules. The corneal epithelium is removed and the lenticule is attached to  
9 the cornea by means of various bonding agents or tissue adhesives, including any suitable  
10 biocompatible adhesive, such as fibrin glue. In an alternative procedure, the preformed collagen-  
11 polymer lenticule is surgically implanted by means of a shallow peripheral groove or slit formed in  
12 the cornea. The lenticule is then shaped by laser ablation to achieve the desired refractive  
13 correction. The corneal epithelium generally grows back over the surface of the lenticule within  
14 three days following the procedure. Growth factors may be incorporated into the composition to  
15 facilitate regrowth of the corneal epithelium and/or healing of the corneal surface if a surgical  
16 incision is required. Various alternative techniques for this procedure are described in U.S. Patent  
17 Nos. 4,923,467 and 5,156,622, incorporated herein by reference.

18 In a similar procedure, following removal of the corneal epithelium, the chemically  
19 modified collagen and activated synthetic polymer are mixed and immediately delivered as a thin  
20 layer (approximately 150 to 200 microns) directly to the surface of the Bowman's layer of the  
21 cornea. The chemically modified collagen and activated polymer covalently crosslink *in situ* to  
22 form a lenticule on the surface of the cornea. Thus formed, the lenticule attaches directly to the de-  
23 epithelialized cornea. Following curing, the material can be laser ablated to achieve the desired  
24 refractive correction, as set forth in U.S. Patent Nos. 5,163,956 and 5,196,027, incorporated  
25 herein by reference. As in the procedure described above using the preformed lenticule, regrowth  
26 of the corneal epithelium occurs within days of the procedure. Again, incorporation of growth  
27 factors into the composition may facilitate regrowth of the corneal epithelium over the lenticule.

28 Due to their mechanical strength and elasticity, the conjugates of the invention are also  
29 useful in a variety of other medical and surgical applications. For example, the chemically  
30 modified collagen-synthetic polymer conjugates may be used as coatings for various medical  
31 devices for long-term incorporation into the body, molded into articles useful for repair or

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1 replacement of cartilage, ligaments, and tendons, or formed into tubes, as described in copending  
2 U.S. application Serial No. 07/985,680, for use as vascular grafts and stents or in nerve repair.

### 3 EXAMPLES

4 The following examples are put forth so as to provide those of ordinary skill in the art with  
5 a complete disclosure and description of how to make the conjugates, compositions, and devices  
6 and are not intended to limit the scope of what the inventors regard as their invention. Efforts have  
7 been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature,  
8 molecular weight, etc.) but some experimental errors and deviation should be accounted for.  
9 Unless indicated otherwise, parts are parts by weight, molecular weight is weight average  
10 molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

#### 11 Example 1

##### 12 (Preparation of PEG Crosslinked Succinylated Collagen)

13 Six (6) liters of collagen-in-solution (CIS) (3 mg/ml collagen in pH 2 HCl) was adjusted to  
14 pH 9 using 0.1 M NaOH at room temperature to produce fibrillar collagen. 1.35 grams of succinic  
15 anhydride powder was added to the fibrillar collagen and the pH maintained between 8.5 and 9,  
16 resulting in the formation of succinylated collagen. The pH of the succinylated collagen was  
17 adjusted to 7.2, then to 4.2 using 0.1 M HCl to precipitate the succinylated collagen. The  
18 succinylated collagen was then centrifuged and the supernatant discarded. The pH of the pellet  
19 was adjusted to 7.2 using 0.1 M NaOH. The succinylated collagen pellet was diluted in water and  
20 the collagen concentration of the resulting succinylated collagen solution determined to be 20  
21 mg/ml.

22 Solutions of difunctionally activated S-PEG in PBS were prepared at different  
23 concentrations as follows: 10 mg S-PEG in 0.1 ml PBS, 20 mg S-PEG in 0.1 ml PBS, 50 mg  
24 S-PEG in 0.1 ml PBS, and 100 mg S-PEG in 0.2 ml PBS. Each of the four crosslinker solutions  
25 was mixed with 0.9 ml of the 20 mg/ml succinylated collagen using syringe-to-syringe mixing.  
26 The four succinylated collagen - S-PEG compositions had final S-PEG concentrations of 10, 20,  
27 50, and 91 mg/ml, respectively. The final collagen concentration of the samples was  
28 approximately 18 mg/ml.

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The four formulations were observed visually for signs of crosslinking at 5 minutes and 2 hours after mixing. As shown in Table 1, the succinylated collagen formulations containing 50 and 91 mg/ml S-PEG showed signs of crosslinking 5 minutes after mixing. All four formulations showed significant crosslinking 2 hours after mixing, forming optically clear gels.

**Table 1. PEG Crosslinking of Succinylated Collagen**

SPEG (mg)	PBS (ml)	Succinylated Collagen (ml)	Signs of Crosslinking? (5 minutes)	Signs of Crosslinking? (2 hours)	Final S-PEG Conc. (mg/ml)
10	0.1	0.9	no	yes	10
20	0.1	0.9	no	yes	20
50	0.1	0.9	some signs	yes	50
100	0.2	0.9	some signs	yes	91

The melting temperatures of the succinylated collagen formulations containing 10, 20, 50, and 91 mg/ml S-PEG (Samples B, C, and D, respectively) were measured using differential scanning calorimetry (DSC) and compared with that of noncrosslinked succinylated collagen (Sample A). DSC is a measure of degree of crosslinking which is commonly used to evaluate gel stability.

DSC results are shown in Figure 1. The melting temperatures for the crosslinked formulations (B, C, and D) were significantly higher than that for the noncrosslinked succinylated collagen (A).

### Example 2

#### (Preparation of PEG Crosslinked Methylated Collagen)

Ninety (90) milliliters of Zyderm® II Collagen without lidocaine (Collagen Corporation, Palo Alto, CA), adjusted to 20 mg/ml collagen concentration, was lyophilized to form freeze-dried collagen. The freeze-dried collagen was then chopped into small pieces.

8.3 milliliters of concentrated hydrochloric acid and 30 grams of sodium sulfate were added to methanol to produce anhydrous acidic methanol. The sodium sulfate was then filtered off of the



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1 anhydrous acidic methanol. Approximately 1 liter of the acidified anhydrous methanol was  
2 subsequently mixed with the chopped freeze-dried collagen.

3 After incubation at room temperature for 7 days, methylated collagen was formed. The  
4 excess methanol was evaporated off. The resulting material was subsequently lyophilized and  
5 dialyzed and the collagen concentration was adjusted to 20 mg/ml by the addition of 0.02 M  
6  $\text{Na}_2\text{HPO}_4/0.13 \text{ M NaCl}$ , pH 7.3.

7 Solutions of difunctionally activated S-PEG in PBS were prepared at different  
8 concentrations as follows: 3 mg S-PEG in 0.15 ml PBS, 9 mg S-PEG in 0.15 ml PBS, 15 mg  
9 S-PEG in 0.15 ml PBS, 30 mg S-PEG in 0.15 ml PBS, 45 mg S-PEG in 0.15 ml PBS, 75 mg  
10 S-PEG in 0.15 ml PBS, 111 mg S-PEG in 0.2 ml PBS, and 165 mg S-PEG in 0.2 ml PBS. Each  
11 of the crosslinker solutions was mixed with 1.35 ml of the 20 mg/ml methylated collagen using  
12 syringe-to-syringe mixing. The methylated collagen - S-PEG compositions had final S-PEG  
13 concentrations of 2, 6, 10, 20, 30, 50, 72, and 106 mg/ml, respectively. The final collagen  
14 concentration of the samples was approximately 18 mg/ml.

15 The resulting formulations were evaluated qualitatively for elasticity and gel strength. As  
16 shown in Table 2, the formulations containing 30 and 50 mg/ml S-PEG showed signs of  
17 crosslinking immediately upon mixing. The compositions containing 2, 6, 10, and 20 mg/ml S-  
18 PEG required approximately 5 to 10 minutes for crosslinking. The compositions containing 72  
19 and 106 mg/ml S-PEG required longer than 10 minutes for gel formation, forming weak, inelastic  
20 gels. The compositions containing between 2 - 20 mg/ml S-PEG resulted in the strongest, most  
21 elastic gels. The composition containing 30 mg/ml S-PEG formed a gel with good strength, but  
22 low elasticity, which could be useful in applications where elasticity is not a desired characteristic.  
23 Compositions of methylated collagen containing greater than 30 mg/ml S-PEG showed poor  
24 elasticity and gel strength. All gels were optically clear.

25 The melting temperatures of the methylated collagen formulations containing 2, 10, 30, and  
26 72 mg/ml S-PEG (Samples E, F, G, and H respectively) were measured using differential  
27 scanning calorimetry (DSC) and compared with that of noncrosslinked methylated collagen. The  
28 DSC results for the noncrosslinked and crosslinked samples are shown in Figures 2 and 3,  
29 respectively. The melting curve profiles indicate that the crosslinked formulations contain a  
30 heterogeneous population of molecules, most of which melt at a higher temperature than the  
31 noncrosslinked collagen.

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As shown in Table 2, the melting temperatures for the crosslinked formulations were significantly higher than that for the noncrosslinked methylated collagen.

Table 2. PEG Crosslinking of 20 mg/ml Methylated Collagen

Final S-PEG Conc. (mg/ml)	Time to Form Gel	Elasticity	Gel Strength	DSC Tm (°C) Range
0	-	-	-	38-43
2	5-10 min.	elastic	good	45-60
6	5-10 min.	elastic	good	-
10	5-10 min.	very elastic	very good	45-68
20	5 min.	slightly elastic	good	-
30	immediate	not elastic	good	47-62
50	immediate	slightly elastic	not good	-
72	>10 min.	not elastic	not good	40-70
106	>10 min.	not elastic	not good	-

### Example 3

*(In vitro* Delivery & Attachment of *in situ* Polymerizable Lenticule to Bovine Cornea)

Methylated collagen having a collagen concentration of 30 mg/ml was prepared as described in Example 2. The epithelial layer of the cornea of an excised bovine eye was removed using a blunt metal spatula. Following de-epithelialization, the cornea was washed with PBS and dried thoroughly using a sponge.

A solution of 10 mg difunctionally activated S-PEG in 0.1 ml PBS was prepared. The crosslinker solution was mixed with 0.9 ml of the 30 mg/ml methylated collagen using syringe-to-syringe mixing. Immediately following mixing, approximately 0.2 ml of the methylated collagen - S-PEG material was extruded from the opening of the 1.0 cc syringe onto the surface of the de-epithelialized bovine cornea.

The methylated collagen - S-PEG material was molded in place on the cornea using a polymethylmethacrylate (PMMA) mold. Crosslinking and gel formation of the collagen-polymer occurred within approximately three minutes to form a lenticule *in situ* on the bovine cornea.

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1           Following gel formation, the mold was removed from the collagen-polymer material. The  
2 surface of the *in situ* formed lenticule was irrigated with PBS. The lenticule was secure and not  
3 dislodged by the irrigation. Gentle teasing of the lenticule with a spatula indicated that it was  
4 favorably attached to the cornea. The lenticule was able to be removed by "peeling" with a spatula.

5           Histological examination (at 100x magnification) was performed on the bovine cornea  
6 before and after removal of the methylated collagen - S-PEG lenticule. Histological examination  
7 before lenticule removal indicated an intimate interface between the lenticule and the cornea.  
8 Following lenticule removal, the surface of the cornea showed no obvious damage or aberrations.

9           The above experiment was repeated using a material prepared from succinylated collagen,  
10 at a 36% level of succinylation and a 30 mg/ml collagen concentration. Twenty (20) milligrams of  
11 difunctionally activated S-PEG was dissolved in 0.1 ml PBS. The crosslinker solution was  
12 subsequently mixed with 0.9 mg of the 30 mg/ml succinylated collagen using syringe-to-syringe  
13 mixing, then delivered to a de-epithelialized bovine cornea. Gel formation occurred within  
14 approximately 10 minutes following delivery of the collagen-polymer material to the cornea.  
15 Qualitative comparison revealed the methylated collagen - S-PEG material to have better attachment  
16 to the cornea than the succinylated collagen - S-PEG material.

17           The present invention is shown and described herein at what is considered to be the most  
18 practical, and preferred embodiments. It is recognized, however, that departures may be made  
19 therefrom which are within the scope of the invention and that obvious modifications will occur to  
20 one skilled in the art upon reading this disclosure.

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1 WHAT IS CLAIMED:

- 1           1.     A conjugate comprising a synthetic hydrophilic polymer covalently bound to a  
2 chemically modified collagen which is in substantially nonfibrillar form at pH 7.
- 1           2.     The conjugate of claim 1, wherein the synthetic hydrophilic polymer is an activated  
2 polymeric glycol.
- 1           3.     A conjugate of claim 2, wherein the activated polymeric glycol is a difunctionally  
2 activated polyethylene glycol.
- 1           4.     The conjugate of claim 1, wherein the chemically modified collagen is succinylated  
2 collagen.
- 1           5.     The conjugate of claim 4, wherein the succinylated collagen has a level of  
2 succinylation within the range of between about 25% and about 50% available lysine residues  
3 succinylated.
- 1           6.     The conjugate of claim 1, wherein the chemically modified collagen is methylated  
2 collagen.
- 1           7.     A conjugate comprising a synthetic hydrophilic polymer covalently bound to a  
2 chemically modified collagen selected from the group consisting of succinylated collagen,  
3 methylated collagen, and mixtures thereof.
- 1           8.     A device comprised of a conjugate of a synthetic, hydrophilic polymer covalently  
2 bound to a chemically modified collagen which is in substantially nonfibrillar form at pH 7.
- 1           9.     The device of claim 8, wherein the device is a lenticule.
- 1           10.    The device of claim 8, wherein the device is a corneal shield.

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- 1 11. The device of claim 8, wherein the device is an artificial corneal implant.
- 1 12. The device of claim 8, wherein the synthetic hydrophilic polymer is an activated  
2 polyethylene glycol.
- 1 13. The device of claim 8, wherein a one millimeter thickness of the article allows  
2 ninety percent or more of the light contacting the article to pass through.
- 1 14. The device of claim 8, wherein the conjugate further comprises a therapeutically  
2 effective amount of a cytokine or growth factor.
- 1 15. A composition comprising:  
2 a conjugate comprising a synthetic, hydrophilic polymer covalently bound to a  
3 chemically modified collagen which is in substantially nonfibrillar form at pH 7; and  
4 a therapeutically effective amount of a pharmaceutically active agent.
- 1 16. The composition of claim 15, wherein the polymer is an activated polyethylene  
2 glycol.
- 1 17. The composition of claim 15, wherein the chemically modified collagen is  
2 succinylated collagen.
- 1 18. The composition of claim 17, wherein the succinylated collagen has a level of  
2 succinylation within the range of between about 25% and about 50% available lysine residues  
3 succinylated.
- 1 19. The composition of claim 15, wherein the chemically derivatized collagen is  
2 methylated collagen.
- 1 20. The composition of claim 15, further comprising a pharmaceutically acceptable  
2 carrier.

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- 1           21.    The composition of claim 20, wherein the pharmaceutically active agent is selected  
2   from the group consisting of an antibiotic, an anti-inflammatory, a growth factor, and a cytokine.
- 1           22.    A process for application of a synthetic lenticule over a cornea of an eye of a human  
2   subject for the purpose of correcting vision, said process comprising the steps of:  
3                removing the outer epithelial layer from the anterior surface of the cornea;  
4                applying a chemically modified collagen, which is in substantially nonfibrillar form  
5   at pH 7, and a synthetic hydrophilic polymer to the exposed corneal surface layer; and  
6                allowing said collagen and said polymer to crosslink *in situ* to form a lenticule.
- 1           23.    The process of claim 22, further comprising the step of profiling the lenticule with a  
2   laser to the extent necessary to effect refractive correction of the patient's vision.
- 1           24.    The process of claim 22, wherein the chemically modified collagen is selected from  
2   the group consisting of succinylated collagen, methylated collagen, and mixtures thereof.
- 1           25.    The process of claim 24, wherein the succinylated collagen has a level of  
2   succinylation within the range of between about 25% and about 50% available lysine residues  
3   succinylated.
- 1           26.    The process of claim 22, wherein the synthetic hydrophilic polymer is an activated  
2   polymeric glycol.
- 1           27.    The process of claim 26, wherein the activated polymeric glycol is a difunctionally  
2   activated polyethylene glycol.

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1           28.    A process for application of a preformed synthetic lenticule over a cornea of an eye  
2 of a human subject for the purpose of correcting vision, said process comprising the steps of:  
3                removing the outer epithelial layer from the surface of the cornea; and  
4                applying a preformed synthetic lenticule comprising a conjugate comprising a  
5 synthetic hydrophilic polymer chemically conjugated to a chemically modified collagen which is in  
6 substantially nonfibrillar form at pH 7.

1           29.    The process of claim 28, wherein the preformed synthetic lenticule is applied to the  
2 surface of the cornea by means of a biocompatible adhesive.

1           30.    The process of claim 28, wherein the preformed synthetic lenticule is applied to the  
2 surface of the cornea by placing said lenticule in a shallow peripheral groove formed in the anterior  
3 surface of the cornea outside the field of vision.

1           31.    The process of claim 28, further comprising the step of profiling the lenticule with a  
2 laser to the extent necessary to effect refractive correction of the human's vision.

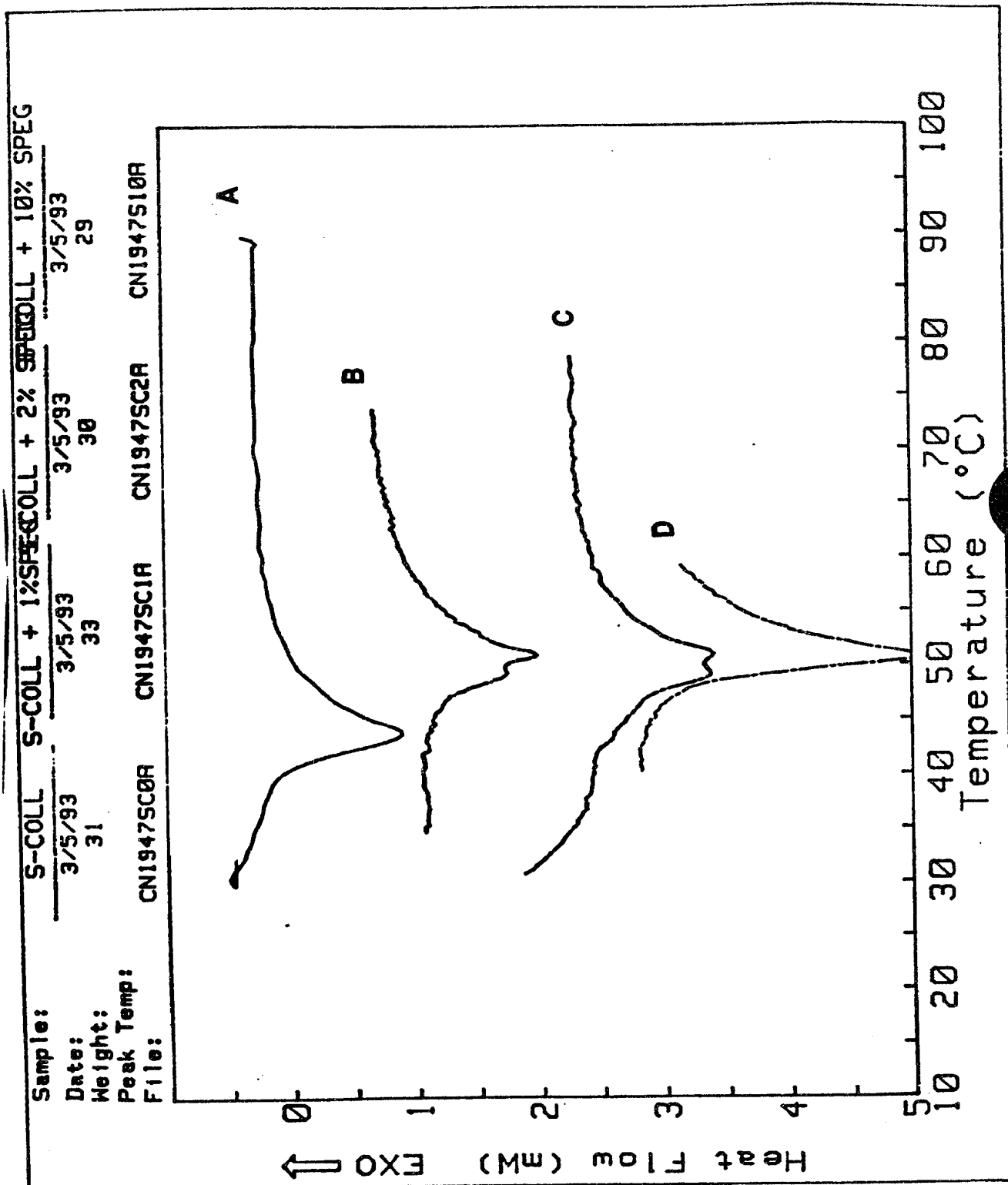
1           32.    The process of claim 28, wherein the chemically modified collagen is selected from  
2 the group consisting of succinylated collagen, methylated collagen, and mixtures thereof.

1           33.    The process of claim 32, wherein the succinylated collagen has a level of  
2 succinylation within the range of between about 25% and about 50% available lysine residues  
3 succinylated.

1           34.    The process of claim 28, wherein the synthetic hydrophilic polymer is an activated  
2 polymeric glycol.

1           35.    The process of claim 34, wherein the activated polymeric glycol is a difunctionally  
2 activated polyethylene glycol.

Figure 1



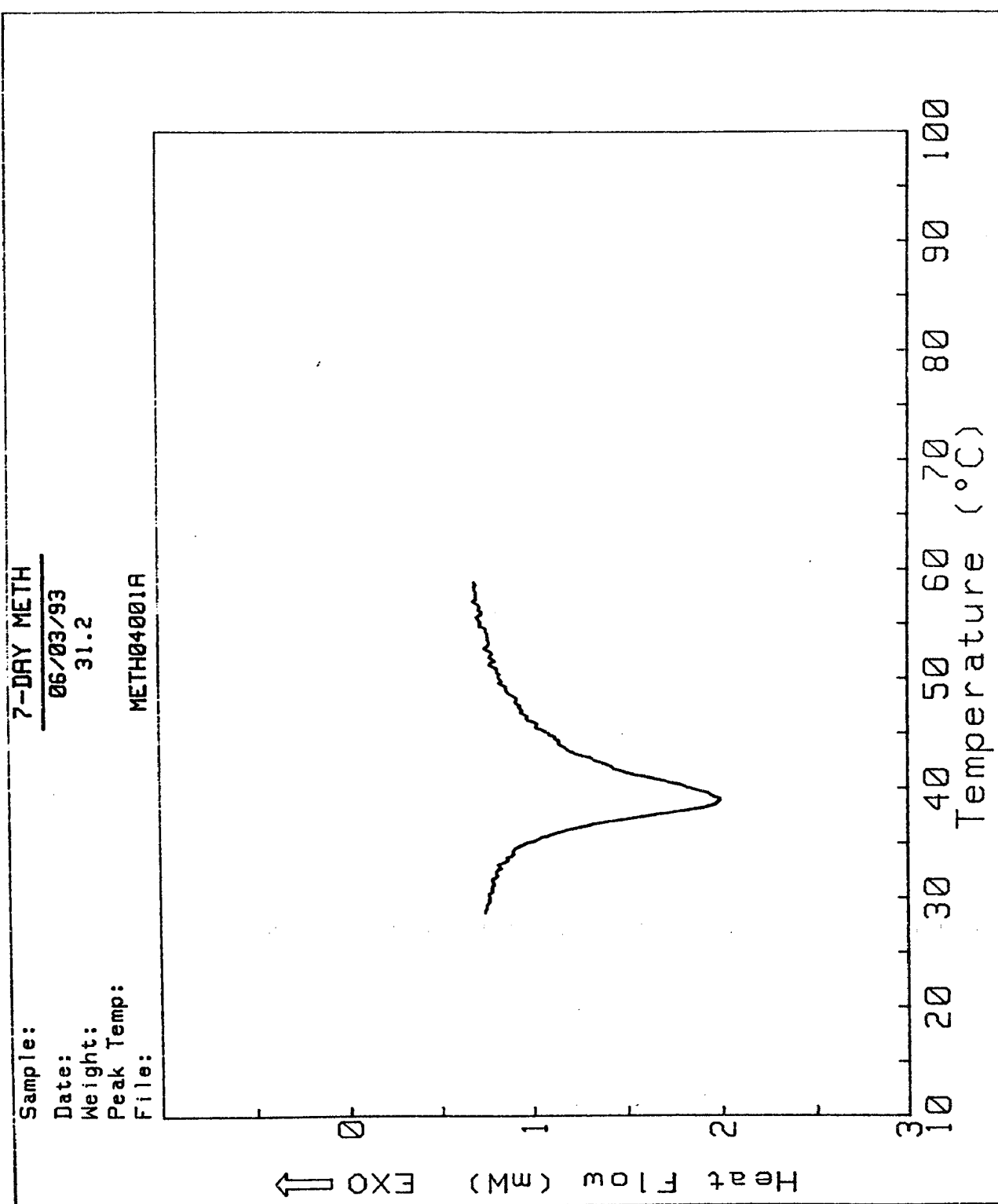
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By:

Cade + Company



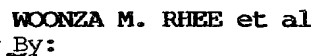
Figure 2



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